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14. ABSTRACT
Epstein Barr virus (EBV) infection in human is associated with variety of malignant diseases including Burkitt's Lymphoma (BL),nasopharyngeal carcinoma, Hodgkin's disease and lymphoproliferative disorders and significant portions of breast cancers. EBV infectioncauses acute infectious mononucleosis but ultimately establishes persistent lifetime latent infection. In all latently infected cells EBVexpresses two small non-polyadenylated RNAs (EBERs). Recent studies have shown that EBERs alone provide tumorigenic potential. Wehave identified that EBERs (which possess extensive secondary structure) has strong nucleotide sequence homology to the coding exon ofkinesin superfamily of motor protein Kif3C. Kinesin is an essential member of the multiprotein ß-catenin degradation complex whichincludes tumor suppressor adenomatos poliposis coli (APC) and GSK3ß. ß-catenin, an activator of Wnt signaling pathway is activated inmany breast cancers. Here we demonstrate that EBER expression in mammary epithelial cell line BT549 induce elevated level of ß-cateninprotein and upregulation of its dependent genes. We also show that in epithelial cells EBER RNA is processed into 19 base small RNA,which is homologous to a 3'-noncoding region of Kif3C mRNA. Further, we demonstrate that Kif3C mRNA is also down-regulated inepithelial cells following EBER expression. Our pilot study thus provides intriguing data that suggests EBER mediated ß-cateninderegulation in epithelial malignancies which possibly takes place via tiny-RNA production from EBERs.

15. SUBJECT TERMS
EBV, non-coding RNA, kinesin, ß-catenin, RNA-interference, epithelial cell growth

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INTRODUCTION

Epstein Barr Virus (EBV) has been detected in significant portion of estrogen negative invasive breast cancers and in large numbers of rapidly growing fibroadenomas of the breast in immunocompromised patients.[1-3] One recent study also detected EBV from estrogen positive breast cancer patients.[4] Because of close association of EBV with various epithelial and lymphoid cancers it has been suggested that EBV plays important role in the genesis of this subset of breast cancers. Epstein Barr virus (EBV) infection in human initially causes acute infectious mononucleosis and later establishes persistent lifetime latent infection. In all latently EBV-infected cells, only a restricted set of EBV genes is expressed. Two EBV-encoded small non-polyadenylated RNAs (EBERs) are constant member of this set. Recent studies have shown that EBERs alone provide tumorigenic potential in EBV negative BL cell lines.[5-8] In recent years several studies, including our own, reported that RNA molecules act as a coactivator or corepressor of gene transcription. Also, small interfering RNAs and micro RNAs are increasingly being identified as important regulator of gene expression. Through BLAST search we have identified that EBERs (which possess extensive secondary structure) has strong sequence homology to kinesin superfamily of motor proteins. Recently, it has been reported that association of kinesin protein KIF3 is required for the transport of tumor suppressor protein adenomatous polyposis coli (APC) along cytoplasmic microtubules.[9] Recent studies have shown that APC, whose mutation was originally identified in colorectal cancers, is also often mutated in breast cancer patients (as high as 18%).[10] APC is known to facilitate proteasomal degradation of ß-catenin, an activator of Wnt signaling pathway that is activated in many breast cancers. Suppression of kinesin has been recently shown to disrupt APC transport and facilitate nuclear accumulation of ß-catenin.[11] *We hypothesize that EBERs down regulate KIF3 protein by RNA interference mechanism and interfere with the functionality and antitumor activity of APC.* The specific aims of this proof of principle study is to analyze if EBERs indeed alter expression of KIF3 protein via RNA interference and to analyze if such alteration provide growth advantages in mammary epithelial cells. Analysis of EBER's role as a tumor suppressor antagonist is highly significant as tumorigenesis is a multistep process, which involves many factors including activation of oncogenes and inactivation of tumor suppressor genes or their products. If our hypothesis becomes true, inhibition of EBERs' expression or their inactivation could be an import therapeutic avenue to treat this subset of breast cancers and the concept may even be used for other EBV associated cancers.

BODY

To fulfill the specific aims of this study we set out six individual tasks as follows:

Task 1. Development of EBER expression system.

Task 2. Analysis of kinesin mRNA and protein expression in cells that are expressing EBV EBER.

Task 3. Analysis of activity of tumor suppressor APC in cells expressing EBER RNA.

Task 4. Identification of small interfering RNA molecules complementary to various kinesins.

Task 5. Analysis of growth properties of cells that are expressing EBER.

Task 6. Data integration and evaluation of the concept hypothesis and pilot experiments to carry the concept ahead.

In this section data obtained so far for individual tasks are presented.

Task 1: Development of EBER expression system

To develop a system for EBER expression in vitro we cloned the EBV genome that contain both the EBER1 and EBER2 coding frames from genomic DNA of EBV positive lymphoid cell line P3HR1. We also PCR amplified individual genes EBER1 and EBER 2.

Primers used for this purpose were (bold bases were enzyme sites and small letters are deviations from original virus sequence):

C: CGGGGTCTC**GGAtCc**CCTAGGTCA
D: TGCCGTTTAATGAT**AGATct**CCAGGAG
1B: CCCGTTTAGGT**aGAtCT**GCGGGATAA
2A: GGGCTTAACGTT**GgATCC**CAGAAGATG

These primers were used according to the figure below (Figure 1) to amplify both EBERs (EBER12 using CD) or individual EBER1 (C-1B) or EBER2 (2A-D) in PCR amplification reaction using high fidelity Taq polymerase. Amplification products were purified, digested with BamHI and BglII (as

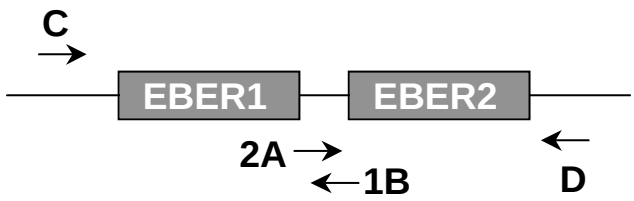

Figure 1. Schematic diagram of the PCR amplification strategy for the EBER1 and EBER2 genes.

appropriate), gel purified and cloned into the BamHI site of pDNA3.1 (Figure 2). The inserts of representative clones were completely sequenced to confirm that there was no PCR introduce error.

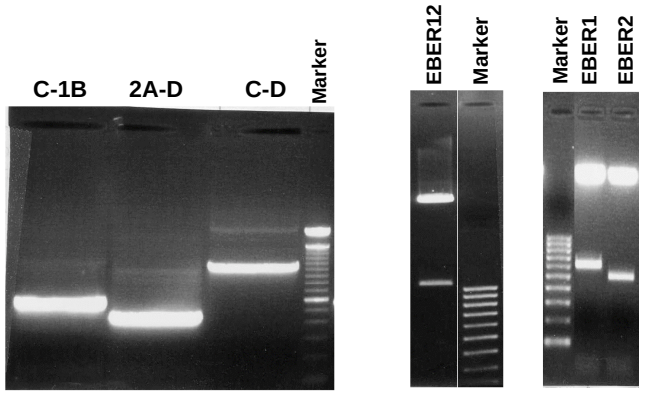

Figure 2. A. Analysis of the PCR amplification product from EBER genes. 100 bp ladder from Invitrogen was used as molecular weight marker. B. Restriction digestion analysis of the pCDNA3.1 clones. 100 bp ladder from Bio-Rad was the marker in this experiment.

To test whether these clones could express EBER RNA, they were transfected in various epithelial cell lines. Total RNA was extracted from transfected cells by Trizol extraction and analyzed by northern blot analysis. EBER1 specific sequence was PCR amplified from pCDNA-EBER1 clone and used as template for the 32P-labeled probe for northern blot analysis. Total RNA preparations from EBV positive and negative Burkitt's lymphoma cell lines (Akata and Ramos, respectively) were used as

positive and negative controls, respectively. As shown in the Figure 3 below, epithelial cells HEK 293, HeLa and MCF-7, all expressed EBER abundantly demonstrating that epithelial cells support EBER production.

Production of EBER in mammary epithelial cell line MCF-10A has not been tested although that in MCF-10F has been successfully performed with the EBER12 plasmid construct. It may be noted that MCF-10A and MCF-10F both comes from same original cell stock (adherent or floating cells) and in vitro they both behave similarly. Therefore, use of MCF-10F in place of MCF-10A is not a deviation from original approved SOW.

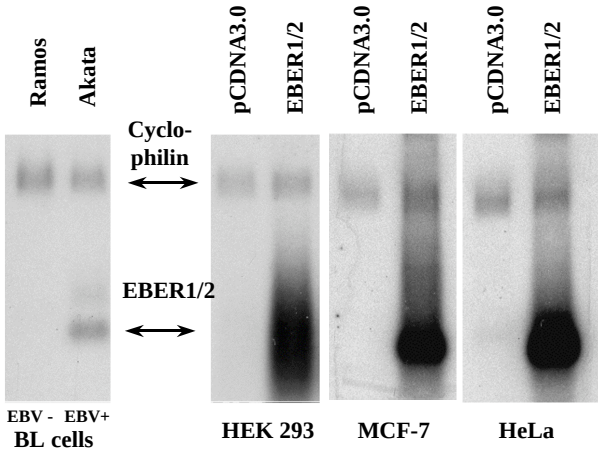

Figure 3. Northern Blot analysis of total RNA from HEK293, MCF-7 and HeLa cells transiently transfected with EBER12 expression plasmid. Total RNA from untransfected BL cells Ramos (EBV-) and AKATA (EBV+) was used as control. Probe for housekeeping gene Cyclophilin was used as a loading control.

Task 2: Analysis of kinesin mRNA and protein expression in cells that are expressing EBV EBER

To determine if Kinesin Kif3 expression level is altered in cells that are expressing EBV EBER, we analyzed Kinesis mRNA level. To get a better assessment of the effect of EBER, we decided to generate cell lines that would stably express EBER and then to test Kif3 mRNA level.

We transfected MCF-7 and HeLa cells with pCDNA-EBER12 clones and maintained them in presence of G418 drug. Several drug resistant colonies were selected, cloned and EBER expression was analyzed by northern blot analysis. As shown in Figure 4, we isolated several G418 resistant colonies

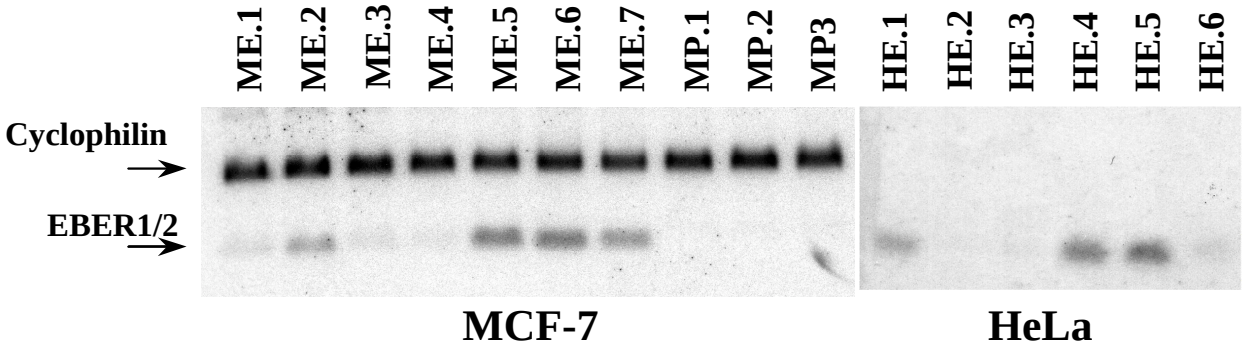

Figure 4. Generation of cell lines that stably express EBER RNA. Total RNA from either MCF-7 or HeLa cells were analyzed for EBER RNA by northern blot analysis. Prefix “ME” denote MCF-7 cells transfected with EBER plasmid and “MP” transfected with vector only. Similarly on the right side panel prefix “HE” denote HeLa cells transfected with EBER plasmid. Position of the EBER and house-keeping gene cyclophilin are indicated on the left. Although cyclophilin probe was not included in northern blot of HeLa cell lines, equal loading was confirmed by ethidium bromide staining of the gel (data not shown).

from all three different cell lines that expressed high level of EBERs.

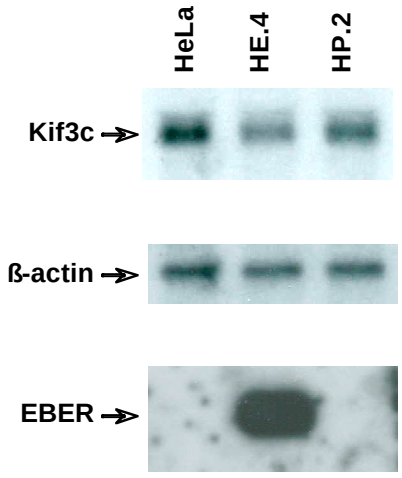

Figure 5. Expression of Kinesin Kif3c mRNA in HeLa cells stably transfected with EBER (HE.4) or with empty vector pCDNA3.1 (HP.2). Twenty microgram of total RNA from each cell was separated in formaldehyde-agarose gel and analyzed by northern blotting using specific probes for Kif3c, EBER or ß-actin.

To test the possibility that EBER expression could affect Kinesin mRNA level, we tested one of the HeLa cell lines we established that stably expresses EBER. We isolated total cellular RNA from one such line HE.4 (see Figure 4 above) and from another HeLa line that was stably transfected with empty vector pCDNA3.1 (HP.2) and tested the Kinesin Kif3 mRNA level by northern blot analysis. A 300 bp KIF3c DNA fragment was amplified by RT-PCR from total RNA isolated from HeLa cells and used as probe for northern blot analysis. Primers used for this purpose was designed from human KIF3c mRNA sequence (genbank accession number NM_002254) forward primer: 5'-GCCCAAGACCTTCACCTTTGA-3', reverse primer: 5'-TTGGAGAGCAGGTCTCGAATC-3'. As shown in Figure 5, Kif3c mRNA level was significantly lower in HE.4 line compared to HP.2 or parental HeLa line. All band intensities were quantified in densitometer and normalized against ß-actin level. The Kif3C mRNA level in HE.4 clone and HP.2 clone was 45% and 85%, respectively, compared to that in normal HeLa cells.

This data demonstrates that EBER expression in HeLa cells lower the Kinesin mRNA level although it remains to be determined yet if this effect is due to RNA interference mechanism.

Task 3: Analysis of activity of tumor suppressor APC in cells expressing EBER RNA

APC is an essential component of ß-catenin degradation complex, which also contain kinesin protein KIF3 and GSK3ß. To test whether EBER expression influence activity of tumor suppressor protein APC we measured ß-catenin accumulation and ß-catenin dependent gene expression in cells following transfection with EBER expression plasmid we constructed. In our preliminary screening of steady state level of ß-catenin by western immunoblotting we found epithelial cell lines HeLa (cervical cancer) and BT-549 (breast cancer) had relatively lower level of ß-catenin compared to other epithelial cell lines we tested such as T47D, HEK 293 and MCF-7. We transfected BT-549 cells with EBER12 plasmid and empty vector pCDNA3.1 and analyzed total ß-catenin in whole cell extracts. As shown in figure 6, EBER transfection increased total ß-catenin level by 2-fold or more compared to vector transfected or untransfected BT549 cells. We also tested whether increased ß-catenin level correlated activation of ß-catenin dependent gene activation. In transient transfection experiment with TCF/LEF dependent reporter plasmid TOP-Flash

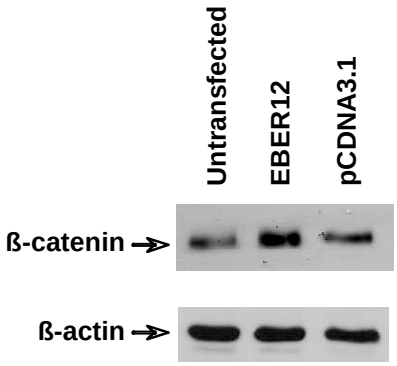

Figure 6. ß-Catenin expression in breast cancer cell line BT-549 following transfection with EBER expression plasmid or the empty vector pCDNA3.1. Whole cell lysates were tested by western immunoblotting using ß-catenin antibody.

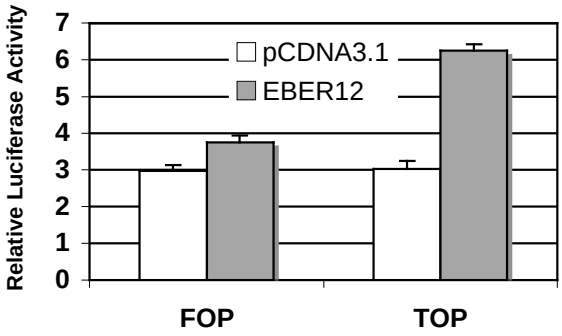

Figure 7. Analysis of ß-Catenin dependent gene expression in breast cancer cell line BT-549 following EBER expression. BT-549 cells were cotransfected with TCF/LEF dependent luciferase reporter plasmid TOP-Flash or a mutant variety FOP-Flash and EBER expression plasmid or the empty vector pCDNA3.1.

which contain 4 TCF/LEF binding sites in the promoter (Upstate Cell Signaling) or a mutant variety FOP-Flash (where all TCF/LEF sites are mutated) we found that EBER expression significantly enhances reporter gene expression in BT549 cells (Figure 7). This data suggested that increase in ß-catenin level due to EBER expression also leads to increase in ß-catenin dependent gene expression in BT-549 cells.

In unstimulated cells, ß-catenin is rapidly degraded in the cytoplasm following its phosphorylation by the degradation complex (which consists of APC, axin/kinesin and GSK3ß) and subsequent ubiquitination and proteasomal degradation. We postulated that EBER expression could degrade Kinesin/Kif3C and render ß-catenin degradation complex non-functional. In such a scenario there will be reduced degradation of ß-catenin. To test this possibility we analyzed the stability of ß-catenin in EBER-expressing cells. BT-549 cells were transfected with vector or EBER expression plasmid and stable-expression was selected using G418 drug resistance. Cells were treated with 20 µM cycloheximide to inhibit new protein synthesis and ß-catenin level in the cells were determined over a period of 4 hrs. Figure 8 shows that the rate of degradation of ß-catenin was appreciably less in EBER-expressing cells compared to vector control cells, as by two hours following cyclohemide treatment, the ß-catenin level in vector transfected cell line was 3-times lower than that of EBER transfected cell line .

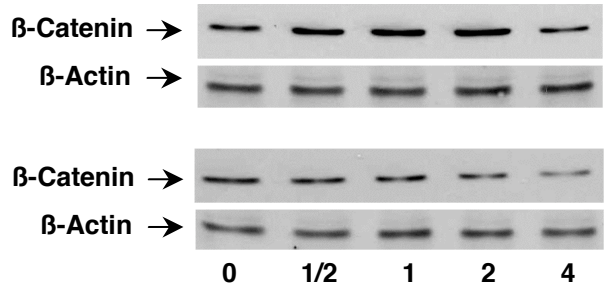

Figure 8. BT-549 cells stably transfected with EBER expression plasmid or the vector (pCDNA3.1) were treated with 20 µM cycloheximide for 0-4 hrs, at which time whole cell lysates were prepared and analyzed for ß-catenin. ß-actin content of the lysates was also measured to confirm equal loading.

Task 4: Identification of small interfering RNA molecules complementary to various kinesins.

The main hypothesis of this Concept Grant application was that EBERs are probably modified into short inhibitory RNA to exert its effect in EBV infected cells. The rationale for this proposition came from the fact that through BLAST sequence search we identified strong sequence homology between

```
106    CTTCCCAGACTCTGCTTTCTGCC  128
       ||||||| ||||||||||||||||
3567   CTTCCCAAACTCTGCTTTCTGCC  3589
```

Homo sapiens kinesin family member 3C
Genbank Accession: BC092406

```
109    CCCAGACTCTGCTTTCTGCC  128
       ||||||||||||||||||||
2898   CCCAGACTCTGCTTTCTGCC  2917
```

Macaca mulatta kinesin family member 3C
Genbank Accession: XM_001084502

Figure 9. Sequence homology of EBER and mammalian Kif3C as identified through BLAST search.

EBER and mammalian kinesin family protein Kif3C (Figure 9). Homology of EBER sequence 106-128 with 3'-noncoding mRNA sequence of human and monkey Kif3C are shown.

To determine if EBER is processed into small RNA homologous to Kif3C mRNA, total RNA extracts from HEK293 cells transfected with EBER expression plasmid were analyzed for microRNAs by Northern Blot analysis. In order to perform this detection process correctly, we followed protocols from the laboratory of David Bartel,[12] who has been a leading scientist in microRNA research (protocol available at web.wi.mit.edu/bartel/pub/protocols_reagents.htm).

As molecular marker to be used in the gel we synthesized 24- and 18-mer RNAs in vitro. We used double-stranded DNA templates with T7 RNA polymerase promoter sequence at the 5'-end and 24 or 18 additional bases ahead of the transcription initiation site. Two custom made oligos with complementary sequences were annealed together to generate these transcription templates. Oligo sequences for the 24-mer were: 5'-TAATACGACTCACTATAGGGTATAGCTGAAACAGCAGAAGT-3' and 5'-ACTTCTGCTGTTTCAGCTATACCCTATAGTGAGTCGTATTA-3'. Oligo sequence for the 18-mer were: 5'-TAATACGACTCACTATAGGGTATAGCTGAAACAGC-3' and 5'-GCTGTTTCAGCTATACCCTATAGTGAGTCGTATTA-3' The 24- or 18-mer RNAs were synthesized in vitro using T7 RNA polymerase and purified by DNase treatment, phenol-chloroform extraction and ethanol precipitation. As positive control for small RNAs with known molecular size we used total RNA extract from cells transfected with commercially available siRNA expression vector. In our experiments we used a plasmid construct that makes 23 base siRNA for mouse toll-like receptor (TLR3) from Invivogen.

Thirty microgram of RNA from 293 cells transfected with either EBER expression plasmid or siTLR3 expression plasmid (positive control RNA) and appropriate amount of molecular size marker were separated by 15% denaturing polyacrylamide gel (containing 6M urea). RNA on the gel was visualized by ethidium bromide staining and transferred onto GeneScreen Plus Nylon membrane (Perkin-Elmer) in a semi-dry electroblotting apparatus. Following UV-cross linking the membrane was prehybridized with UltraHyb Oligo solution (Clontech) at 42°C for 30 min and then hybridized overnight at 42°C in the same solution with oligo probe. A negative strand oligo complementary to both 24-mer and 18-mer RNA marker (5'-ACTTCTGCTGTTTCAGCTATACCC-3'), positive strand oligo complementary to siTLR3 RNA (5'-GAGCATCAATCTAGGACTGAA-3') and negative strand oligo

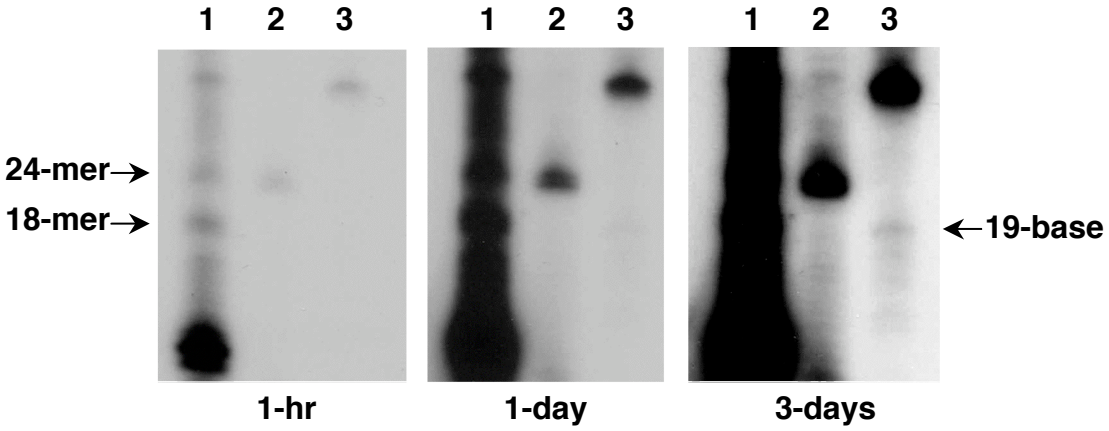

Figure 10. Detection of EBER derived microRNAs by Northern hybridization. Lane 1, RNA size markers generated by in vitro transcription as indicated in the text; lane 2, total RNA from 293 cells transfected with siRNA-TLR3 construct; lane 3, total RNA from 293 cells transfected with EBER expression plasmid. The blot was hybridized with a mixture of three individual probes as indicated above. Exposure time for each individual X-Ray films are indicated at the bottom

complementary to EBER sequence 106-128 (5'-GGCAGAAAGCAGAGTCTGGGAAG-3') were end labeled with ^{32}P-γATP. Each of these three probes was mixed in the same hybridization mixture. Following hybridization, the membrane was washed in non-stringent wash buffer (3% SSC, 5% SDS) for 20 min (twice, 10 min each) and stringent wash buffer (1% SSC, 1% SDS) for 5 min and exposed to Kodak BioMax films for appropriate duration of time.

As shown in Figure 10, we were able to clearly identify a 23-base RNA in total RNA from 293 cells transfected with siRNA-TLR3 construct (lane 2). Interestingly, a band corresponding to approximately 19 bases was detected in total RNA from EBER-transfeced 293 cells. However, the intensity of this signal was significantly lower than that observed for siRNA-TLR3 and was visible only in longer exposure of the blot. In other experiments we demonstrated that positive strand siTLR3 RNA probe or the negative strand 24-mer probe does not hybridize with EBER RNA or the corresponding micro RNA (data not shown). This experiment thus demonstrated that a ~19 base EBER-specific microRNA is indeed made in cells those express full-length EBER RNA.

Task 5: Analysis of growth properties of cells that are expressing EBER.

We hypothesized that expression of EBER would inhibit kinesin expression by RNA interference and consequently block degradation of ß-catenin. Increased amount of ß-catenin would then lead to enhanced expression of ß-catenin dependent genes and ultimately provide proliferative signals to the cell. To test this hypothesis, in our original statement of work we proposed to perform cell proliferation assay in mammary epithelial cell line MCF-10A (or MCF-10F) and in breast cancer cell line MCF-7 that are stably transfected with EBER. Unfortunately, our repeated attempt to establish MCF-10F line stably expressing EBER was unsuccessful. Transfection efficiency was always too low and cells did not survive G418 drug selection.

However, we performed cell proliferation assay with breast cancer cell line MCF-7. We used

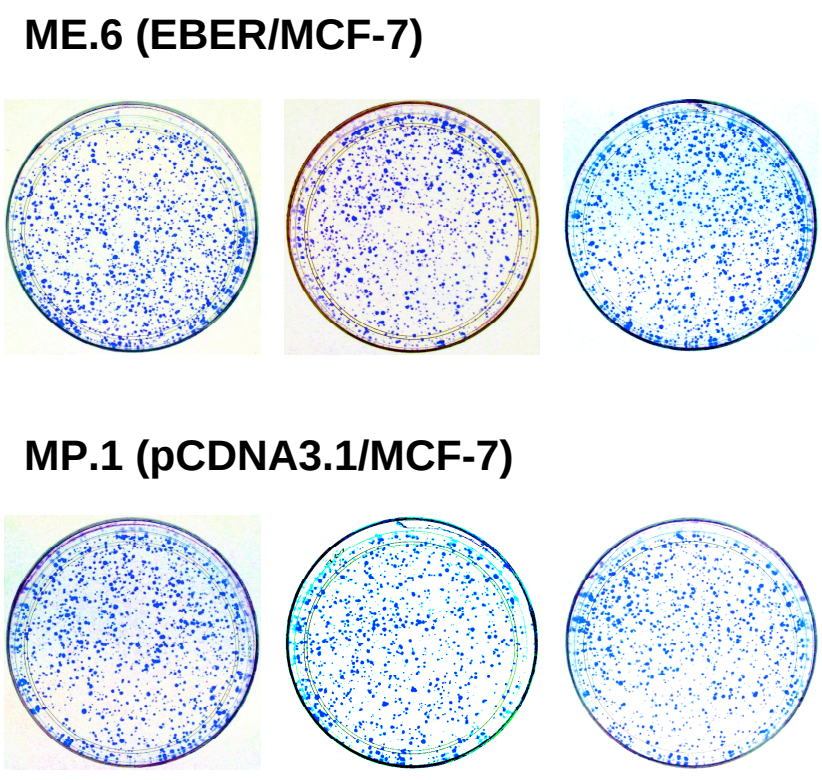

Figure 11. EBER expression does not provide cell proliferative signal to MCF-7 cells. MCF-7 cells stably transfected with either EBER or backbone vector pCDNA3.1 were plated at a low density (1000 cells in 100 mm plates) and allowed to grow for two weeks. Cells were Giemsa stained, colonies counted and photograph taken against fluorescent light.

MCF-7 line stably transfected with EBER (please see figure 2. ME.6) and with vector pCDNA3.1 (MP.1, figure 2). We plated 1000 actively growing cells of both lines in 100 mm tissue culture dishes (in triplicate) and allowed to grow and form colonies over two weeks time. At the end of this 2-week period, number of colonies and their size for each clone were compared. As shown in Figure 11, no statistically significant difference in the size or the number of colonies between cells expressing EBER or the plasmid vector was observed. This data suggested that at least in MCF-7 breast cancer cell line EBER expression does not provide any growth advantage.

Task 6: Data integration and evaluation of the concept hypothesis and pilot experiments to carry the concept ahead.

In this concept grant application our objective was to test whether EBERs interfere with the function of tumor suppressor APC in epithelial cells. The rationale for such a study was that improper function of APC is associated with various cancers. If EBERs indeed interfere with normal functions of APC, this could be a mechanism by which EBERs influence epithelial malignancy. The other aspect of this application was to test the idea that small interfering RNA is possibly made from EBERs that degrade a protein (Kif3C) required for proper function of APC.

We have been able to demonstrate that the function of APC (as measured by ß-catenin regulated gene transcription) is indeed deregulated in epithelial breast cancer cell line BT549 (Task 3). Inefficient function of APC cannot degrade cytoplasmic ß-catenin properly and allows ß-catenin to migrate to nucleus and upregulate specific gene expression.

Our study on expression of small RNAs in cells expressing EBER in Task 4 demonstrated that RNA species of approximately 19 bases are made in epithelia cells such as HEK293. We also demonstrated that EBER expression leads to decrease in Kif3C mRNA level (Task 2). We however, did not get a clear answer to whether EBERs helps directly in epithelial cell proliferation (Task 5).

In our view the work from this concept grant provided evidence that EBER makes small RNA in epithelial cells, which is similar in sequence to Kif3C, and EBER expression leads to diminution of Kif3C mRNA level. This decrease is possibly associated with failure of APC/Kinesin/GSK3ß mediated ß-catenin degradation. This information is significant both with respect to the mechanism of EBER function as well as role of EBV in EBV-associated epithelial cancers. However, there are several areas as elucidated in the “conclusion” section that needs in-depth study.

KEY RESEARCH ACCOMPLISHMENTS

- The non-polyadenylated small RNAs from Epstein-Barr virus (EBER) is readily expressed in all epithelial cells without the need of any external promoter. This was observed in transiently transfected cells as well as in stable-expression cell lines.

- EBER expression increased the stability of ß-catenin and its steady state level in breast cancer cell line BT549. EBERs also upregulated the expression of ß-catenin dependent genes in these cells.

- Expression of mRNA of kinesin protein Kif3C was found to be significantly lower in epithelial cell line HeLa that was stably transfected with EBER.

- Small, approximately 19-base RNA processed from EBER, with matching sequence to human Kif3C was identified in epithelial cells HEK293 transfected with EBER.

- We were unsuccessful in establishing stable EBER-expressing mammary epithelial cell line (MCF-10F) and could not test the role of EBER in the growth of those cells. Cell proliferation assay in breast cancer cell line MCF-7 however, showed that EBER expression did not provide them with any added growth advantage.

REPORTABLE OUTCOMES

A.
Data obtained from this work was presented as a Poster in the "Era of Hope" Breast Cancer Meeting organized by Department of Defense, which was held in Pennsylvania Convention Center, Philadelphia, June 8-11, 2005.

Title: ROLE OF EPSTEIN-BARR VIRUS ENCODED SMALL RNAS IN BETA-CATENIN DEGRADATION
Authors: Sajal K. Ghosh and Idowu Akinsheye
Abstract:
Significant improvement of breast cancer survival rate in recent years has been possible because of our better knowledge of the risk factors for the disease and molecular mechanism of the abonormalities as well as, early detection. In recent years, Epstein Barr Virus (EBV) has been detected in significant portion of estrogen receptor negative invasive breast cancers. EBV has also been detected in large numbers of rapidly growing fibroadenomas of the breast in immuno-compromised patients. EBV is associated with variety of malignant diseases including Burkitt's Lymphoma (BL), nasopharyngeal carcinoma, Hodgkin's disease and lymphoproliferative disorders of immunosuppressed patients. Because of this close association it has been suggested that EBV plays important role in the genesis of EBV associated breast cancers. Although most of the EBV genes are not expressed in EBV associated tumors, two non-coding RNA molecules (EBERs) are expressed consistently. Recent studies have suggested that these EBERs may have oncogenic potential. We have identified that EBERs (which possess extensive secondary structure) has strong sequence homology to kinesin superfamily of motor proteins. Suppression of kinesin protein (KIF3C) has been recently shown to disrupt adenomatous polyposis coli (APC) transport and facilitate nuclear accumulation of beta-catenin. ß-Catenin is an important activator of Wnt signaling pathway that is activated in many breast cancers. We hypothesize that EBERs down regulate KIF3 protein by RNA interference mechanism and interfere with the functionality and antitumor activity of APC.
In order to test our hypothesis, we have cloned the EBER genes in pCDNA 3.1 vector. In transient transfection experiments in epithelial cells such as HeLa, human embryonic kidney cells 293 and also in human breast cancer cell line MCF-7, followed by northern blot analysis, we confirmed that these constructs make large amount of EBERs. Next, we analyzed KIF3 gene expression in EBER expressing cells by northern blotting. A 350 base pair cDNA segment of KIF3 mRNA was amplified from total cellular RNA from HeLa cells and was used as probe for this purpose. Our preliminary data indicate that KIF3C mRNA level was significantly lower in HeLa cells that were expressing EBER but not in cells that were transfected with empty pCDNA vector. We are currently testing how this apparent reduction in KIF3C level affects ß-catenin degradation in a transient ß-catenin dependent reporter assay system. Using commercially available antibodies, we are also analyzing KIF3 protein expression in cells that

are expressing EBER. Analysis of EBER's role as a tumor suppressor antagonist is highly significant as tumorigenesis is a multistep process which involve many factors including activation of oncogenes and inactivation of tumor suppressor genes or their products. Detail results of these studies will be presented in the meeting.

B.
A NIH small grant application (R03) entitled "ß-CATENIN ACTIVATION IN EBV LATENCY" has been made by the PI based on the finding of this concept grant.

C.
A manuscript with tentative title "Epstein Barr virus encoded small RNAs facilitate ß-catenin stabilization" is in preparation.

CONCLUSIONS

This concept grant provided us with nice opportunity to test our hypothesis that small interfering RNAs are possibly made from EBV encoded RNAs (EBER) and may be responsible for EBER's role in EBV associated mammary epithelial tumors. We hypothesized because of sequence similarity of EBER with kinesin Kif3C, interfering RNAs against Kif3C is probably made in cells that express EBER. As outlined above, we clearly demonstrated that EBER expression reduces Kif3C mRNA level in HeLa cells. We also demonstrated that ß-catenin is relatively stable in cells that express EBER over EBER-negative cells and there is a consequent upregulation of ß-catenin dependent gene expression.

The Wnt/ß-catenin signaling pathway plays an important role in tissue development and as such it is strictly regulated. Any mutation that constitutively activates this pathway could therefore involve in initiation and progression of cancer. In fact, multiple *in vivo* and *in vitro* studies already implicated aberrant ß-catenin signaling as the determining factor in variety of cancers[13-15]. Mutation in the ß-catenin gene (*ctnnb1*) has been documented in the cancers of colon[16,17], prostate[18], ovary[19], liver[20], lung[21], skin[22], uterine endometrium[23,24] and urinary bladder[25] among others. Studies based on these presumptive links indeed show that ß-catenin is activated in latent EBV infection. In one study EBV latent protein LMP2A directly activated ß-catenin signaling in telomerase-immortalized human foreskin keratinocytes[26]. Our data shows that EBV latent gene product EBERs (the non-coding RNAs) also activates ß-catenin dependent gene expression. It is noteworthy that usurpation of ß-catenin pathway by several other human pathogenic viruses has also been documented in recent years[27-31]. As ß-catenin stabilization has been demonstrated in BL cells, which displays type 1 latency and expresses only EBNA1 and EBERs[32], it is possible that EBV gene products other than LMPs may also have a role in ß-catenin activation. Our data that EBERs independently activate ß-catenin signaling is thus highly significant and deserves to be tested thoroughly.

We have demonstrated EBER-mediated inhibition of ß-catenin degradation and subsequent gene upregulation in only BT549 cells which is a breast cancer cell line. It will be necessary in future experiments to test whether other breast cancer cell lines and mammary epithelial line behave similarly in response to EBER expression. Since the level of ß-catenin is often elevated in cancer cells, it is possible that many of the breast cancer cell line may have already elevated ß-catenin level. In choosing other breast cancer lines to test EBER function we have to consider that. Further, it will be very useful to test whether those cells support production of additional amount of ß-catenin and associated gene activation by introducing ß-catenin protein expression vector into these cells. BT-549 cells do have

lower level of ß-catenin compared to other breast cancer lines such as MCF-7 and T47D. Our observation that EBER enhances ß-catenin mediated gene transcription is thus a genuine effect.

Analysis of RNA preparations from cells expressing EBER provided evidence that EBERs are processed into small RNAs which potentially could interfere with expression of cellular mRNAs such as that of Kif3C. We used a 23 base negative strand EBER probe that had sequence homology to Kif3C mRNA. Because we found a distinct band of approximately 19 bases, it is unlikely that this was part of randomly broken down piece of the 172-base EBER RNA. However, it is noteworthy that the amount of this product was very low compared to EBER expression level and was detected in 293 cells, which is highly transfectable. We will need to test whether this small RNA can also be detected in other epithelial cells, in particular, those cells where EBERs downregulate ß-catenin degradation or where Kif3C mRNA level are down because of EBER expression. It may be noted that the sequence of EBER that is homologous to Kif3C is in positive orientation. However, this stretch of EBER sequence also forms hairpin structure in RNA folding prediction analysis (by M-fold program). Thus the other strand of the hairpin can actually act as the interfering antisense RNA for the Kif3C mRNA. This idea need to be tested in future experiments.

We found reduction of Kif3C mRNA level due to EBER expression in one stably transfected HeLa cell line. In future studies we must test other EBER stable lines to establish clearly the effect of EBER on Kif3C mRNA degradation. We have been unsuccessful in determining the role of EBER in proliferation mammary epithelial cells during the course of this study. We are continuing our attempt to test mammary epithelial cell line MCF-10F for this purpose with better transfection technique.

In summary, data generated from this concept grant clearly suggest that EBERs are processed into small RNAs, which potentially could upregulate ß-catenin mediated gene regulation via downregulation of ß-catenin degradation. A recent study published in May 2006 demonstrated that decrease in ß-catenin expression in breast cancer patients is associated with poor prognosis.[33] Our results are thus are intriguing preliminary data to launch a more comprehensive study on the role of EBER in ß-catenin regulation which has clear potential to shed light on EBV's role in not only breast cancers but also other epithelial malignancies.

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APPENDICES

None

SUPPORTING DATA

None

LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Sajal K. Ghosh